WADA Technical Document – ISL TD2027ENDO

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Written by: Reviewed by:	*WADA* Science/Endocrine Working Group *WADA* Endocrine ABP WG/ Laboratory Expert Advisory Group	Approved by:	*WADA* Executive Committee
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Analytical and Reporting Requirements for the Blood *Markers* of the Endocrine Module of the *Athlete Biological Passport*

1.0 Introduction

The purpose of this *Technical Document* (*TD*), which constitutes an integral part of the *International Standard* for Laboratories (ISL) [1] is to harmonize the analysis and reporting of the blood (serum) *Markers* of human Growth Hormone (hGH) as part of the Endocrine Module of the *Athlete Biological Passport* (*ABP*) to uncover the *Use* of *Prohibited Substances* associated with hGH biological activity, namely recombinant hGH preparations, hGH secretagogues, hGH analogs and releasing factors, and Insulin-like Growth Factor-I (IGF-I).

1.1 Procedure for Analysis of the Blood *Markers* of the Endocrine Module

The Analytical *Testing* Procedure (ATP) involves the measurement of the serum concentrations of two *Markers* of hGH biological activity, namely IGF-I and N-terminal Pro-peptide of Type III Collagen (PIIINP), which are naturally present in blood and whose concentrations are increased following hGH administration. The measured concentrations of these two *Markers* are then combined in a score calculated automatically in *ADAMS* (*i.e.*, the GH-2000 score), which is sex-specific and includes an adjustment for age to reflect the age-related decline in hGH and *Marker* concentrations.

a) The ATP for the blood endocrine *Markers* is not a mandatory ATP (see ISL *TD* ATP [2]) and is not applied to all serum *Samples*. Therefore, these blood endocrine *Markers* shall be measured in serum *Sample*(s) (see Article 2.0) by Laboratories with appropriate analytical capacity and upon request by the responsible *Testing* Authority (TA) or *WADA*, and results shall be reported in *ADAMS*.

b) The analysis of the blood endocrine *Markers* follows a two (2)-step procedure:

   i. An Initial *Testing* Procedure (ITP) based on the quantification of intact IGF-I by top-down Liquid Chromatography-(tandem) Mass Spectrometry (LC-MS^n) or High-Resolution Mass Spectrometry (LC-$HRMS^n$), and on the quantification of P-III-NP using the Siemens ADVIA Centaur P-III-NP chemiluminescence sandwich immunoassay (Siemens Healthcare Laboratory Diagnostics, Camberley, UK), and

   ii. A subsequent Confirmation Procedure (CP) may be performed, which consists of the same assay pairing used for the quantification of the concentrations of IGF-I and P-III-NP in the ITP, as well as the identification of IGF-I (in compliance with the ISL *TD* IDCR [3]). A CP shall be performed when at least one of the three blood endocrine *Markers* (IGF-I, P-III-NP, or GH-2000) in the *Sample* constitutes an outlier in the corresponding Passport for elevated values, as determined by the Adaptive Model, triggering an Endocrine – Confirmation Procedure Request ("Endocrine-CPR") in *ADAMS*. A CP may also be performed upon request to the Laboratory (see Article 4.2).

If an outlier for elevated IGF-I from the ITP is confirmed, a subsequent analysis will be performed on the *Sample* applying the hGH Isoform Differential Immunoassay (see ISL *TD* GH [4]).

2.0 Assay Pre-analytical Procedure

a) The Laboratory should (usually) receive refrigerated (not frozen[1]) "A" and "B" blood *Samples*, which have been collected in serum tubes containing an inert polymeric serum separator gel and a clotting activator (for example: BD Vacutainer® SST™-II Plus tubes, EU ref 367955; BD Vacutainer® SST™-II Plus Advance tubes, EU ref 367954; BD Vacutainer® SST™ tubes, US ref 367986) in accordance with the *International Standard* for *Testing* (*IST*) [5]. The use of alternative collection devices shall be validated by the relevant Laboratory(ies) and approved by *WADA* prior to use for *Sample* collection.

b) Alternatively, if the clotting and centrifugation of the blood *Sample* is performed prior to reception at the Laboratory (for example, at the site of *Sample* collection) or when a blood *Sample* is shipped from another Laboratory for subcontracted analyses, *Samples* may be received at the Laboratory as frozen/refrigerated *Samples* either in the same *Sample* collection tubes or as separated serum in new tubes.

c) The Laboratory shall check the status of the *Sample*(s) and the integrity of the collection tubes (*e.g.,* evidence of breakage of the separating gel). The Laboratory shall note any unusual condition of the *Sample* and record such condition(s) in the Lab Results in *ADAMS*.

d) Any *Samples* delivered to the Laboratory in tubes containing an anti-coagulant (for example, whole blood *Samples* collected in EDTA tubes), or as separated plasma, shall not be analyzed for the blood hGH *Markers*.

e) The Laboratory shall notify and seek advice from the TA regarding rejection or Analytical *Testing* of *Samples* for which irregularities are noted (see ISL [1]).

[1] Unless the blood matrix components have been separated before shipment to the Laboratory.

2.1 Blood *Samples* Received as Non-separated in Serum Tubes

Sample Processing upon Reception	– Both "A" and "B" *Samples* shall be centrifuged for 10-15 min at 1300-1500 g as soon as possible after reception at the Laboratory. – "A" *Sample* If the "A" *Sample* is not opened to be analyzed within five (5) days from *Sample* collection, then the Laboratory may: • Keep the centrifuged "A" *Sample* in the *Sample* collection tube and step-freeze it (at approx. -15°C or less and according to the tube manufacturer's instructions) until thawing and aliquoting for analysis, or • Aliquot the separated serum fraction into new vials (ensuring that appropriate Laboratory Chain of Custody (see ISL *TD* LCOC) [6] is maintained), which shall be stored frozen (at approx. -15°C or less) until thawing for analysis. – "B" *Sample* The centrifuged "B" *Sample* shall be step-frozen and stored (at approx. -15°C or less and according to the tube manufacturer's instructions) until use, if needed (see below). *[Comment: If the Laboratory transfers the Aliquot into new vials for frozen storage, the vials should ensure proper sealing for optimal storage (cryovials with an "O-ring"). Thawing of Sample(s) for analysis should be done stepwise; Samples shall not be thawed under hot water or any other similar process that risks raising the temperature of the Sample above room temperature. Thawing overnight under refrigeration (2-8 °C) is recommended.]*
Sample Processing for Analysis	a) ITP An Aliquot of the "A" *Sample* serum fraction shall be taken for the ITP of the blood endocrine *Markers*, and shall be processed as follows: • It may be analyzed immediately after aliquoting; or • It may be stored refrigerated (2-8 °C) if analyzed within a maximum of five (5) days from *Sample* collection; or • It shall be stored frozen (at approx. -15 °C or less) if the analysis will be conducted more than five (5) days from *Sample* collection. The remaining "A" serum fraction may be kept in the *Sample* collection tube or aliquoted into new vial(s) and shall be stored frozen (at approx. -15 °C or less) if the analysis will be conducted more than five (5) days from *Sample* collection[2]. b) CP The CP shall be performed on a new Aliquot of the remaining "A" *Sample* serum fraction and shall be conducted immediately after aliquoting.

[2] It is recommended that the Laboratory stores the serum *Samples* frozen (at approx. -70 °C or less) if the TA (or *WADA*) has requested the Laboratory to place them into long-term storage (> 3 months) for Further Analysis purposes (see also ISL Article 5.3.7.2).

2.2 Blood *Samples* Received as Centrifuged and Frozen/Refrigerated

Sample Processing upon Reception	a) If *Samples* are received frozen, they shall remain frozen until thawing and aliquoting for analysis. b) If *Samples* are received refrigerated, the "A" *Sample* should be processed to obtain an Aliquot for analysis as soon as possible (as per Article 2.1.), while the "B" *Sample* shall be stored frozen (at approx. -15 °C or less) until aliquoting for analysis.
Aliquot, Storage and Analysis	a) ITP i. Once a serum Aliquot of the "A" *Sample* is taken for the ITP of the blood endocrine *Markers*, it should: • Be analyzed immediately or may be stored refrigerated (2-8 °C) if analyzed within a maximum of five (5) days from *Sample* collection; or • Stored frozen (at approx. -15 °C or less) if the analysis is to be conducted after five (5) days from *Sample* collection [3]. ii. The remaining "A" serum fraction shall be stored as per Article 2.1 above. b) CP The CP shall be performed on a new Aliquot of the remaining "A" *Sample* serum fraction and shall be conducted immediately after aliquoting.

[Comment to Articles 2.1 and 2.2: When analyses specific to the ABP are requested, only the "A" Sample shall be considered for the ITP and CP. In cases where the "A" Sample is not suitable for the performance of the ABP Markers analysis (e.g., there is insufficient Sample volume; the Sample container has not been properly sealed or has been broken; the Sample's integrity has been compromised in any way; the "A" Sample is missing), a splitting procedure of the "B" Sample could be performed, as detailed in the ISL [1].]

3.0 Analytical *Testing* Procedure Validation and Analysis Requirements

For the implementation of the ATPs for the analysis of the blood endocrine *Markers* of the *ABP* in routine *Doping Control* analysis, the Laboratory shall fulfil the following requisites:

a) Validate the Quantitative Procedures (for ITP and CP) for measuring the *Marker* concentrations, as well as the Qualitative Procedure (for CP) for IGF-I identification, as per the ISL *TD* VAL [7] requirements.

b) The validated ATPs shall meet the acceptance values for the parameters of assay performance applicable to the separate quantification of IGF-I and P-III-NP concentrations as specified in Article 3.1 (as applicable).

c) The Laboratory shall apply the validated ATPs in accordance with the ATP Analysis Requirements specified in Article 3.2.

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3.1 ATP Validation Requirements (see also ISL *TD* VAL [7])

Endocrine *Marker*	IGF-I	P-III-NP
Identification of *Markers*	The Laboratory shall validate the Qualitative Procedure for confirmation of the identity of IGF-I in accordance with the requirements of the ISL *TD* IDCR [3] and ISL *TD* VAL [7]	N/A
Limit of Quantification (LOQ)	≤ 50 ng/mL	≤ 1 ng/mL
Working Range	50 -1000 ng/mL	1 – 20 ng/mL
Relative Standard Combined Measurement Uncertainty, u_c (%),	(≤) 30% at LOQ (≤) 20% at > 150 ng/mL	≤ 15%

3.2 ATP Analysis Requirements

Endocrine *Marker*	IGF-I	P-III-NP
Test Method and Instrumentation	Quantitative Procedure (ITP and CP): Top-down (intact) Liquid Chromatography combined with tandem Mass Spectrometry based on triple quadrupole (LC-MS^n) or High-Resolution Mass Spectrometry analyzer (LC-HRMS). Qualitative Procedure (CP): LC-MS^n (or LC-HRMS) (in compliance with the ISL *TD* IDCR [3])	Quantitative Procedure (ITP and CP): Siemens ADVIA Centaur P-III-NP chemiluminescence immunoassay
Aliquot	Shall be measured (ITP and CP) in singlicate (1x) on one (1) serum Aliquot not greater than (≤) 50 µL	Shall be measured (ITP and CP) in singlicate (1x) on one (1) serum Aliquot according to manufacturer's instructions.
Internal Standards	Stable isotope-labeled IGF-I (*e.g.,* NIST or ProSpec ^{15}N-IGF-I).	N/A
Calibration	A freshly prepared Single Point Calibrator (SPC) shall be included in each analytical batch. The recombinant human IGF-I calibrator from NIST (SRM 2926) should be used to prepare the SPC. Any other calibration material shall be validated against the NIST SRM 2926 calibrator.	As per kit instructions

Quality Controls	The QCs shall be prepared either from authentic serum or by spiking a standard solution(s), independent from that used for the calibrator(s), into serum. Following preparation, all QC material shall be aliquoted and stored frozen (preferably at approx. -70 °C or less for long-term storage) until use. At least one (1) serum QC sample representative of the low part of the working range (*e.g.*, at a concentration within the first quartile of the working range) and one (1) serum QC sample representative of the high part of the working range (*e.g.*, at a concentration within the fourth quartile of the working range) shall be used. The QCs may be prepared independently for each *Marker* or containing both *Markers* at the appropriate concentrations in a single QC sample. For the CP, at least one QC sample, depending on the initial quantification results for the *Markers*, shall be included in each confirmatory analytical batch.

4.0 Confirmation Procedure for the Endocrine Module

4.1 Confirmation Procedure Requests for Blood Endocrine *Markers* Triggered through *ADAMS*

a) Once the ITP data of the blood endocrine *Markers* is entered and matched with the corresponding *Doping Control* Form (DCF) *in ADAMS*, the Adaptive Model automatically updates the endocrine Passport. If an outlier is identified based on an abnormally high GH-2000 score, IGF-I, and/or P-III-NP value, an Endocrine-CPR is triggered and sent automatically to the Laboratory through *ADAMS*. The Laboratory shall ensure the reception and management of CPR notifications using a dedicated *ADAMS* account(s).

b) The TA[3] shall inform the Laboratory whether to proceed or not with the CP of the blood endocrine *Markers*, within fourteen (14) days from the receipt of the Endocrine-CPR notification.

i. Upon receipt of confirmation to proceed with the CP, the Laboratory shall proceed with the CP of the blood endocrine *Markers* as soon as possible.

ii. Any justification from the TA or the PC[3] to not proceed with the CP shall be provided in writing according to Article 8.6 of the ISL *TD* APMU [8]. In such cases, the Laboratory shall update the Lab Results in *ADAMS* for the *Sample* with a comment stating that the TA or the PC[3], as applicable, requested to not perform the CP, and the reasons given.

iii. In the absence of communication from the TA or the PC[3] within fourteen (14) days from the Endocrine-CPR notification, the Laboratory shall proceed with the CP of the blood endocrine *Markers*.

[3] The APMU or PC, where the PC is not the TA, may contact, in writing, the Laboratory regarding performance of a CP of the blood endocrine *Markers* on behalf of the TA. In such cases, the APMU (which may have been bestowed such authority by the PC) or the PC shall copy the relevant TA.

c) When the Laboratory receives an Endocrine-CPR for a *Sample* for which *Adverse Analytical Finding*(s) (*AAF*) have been reported for other *Prohibited Substance*(s) or *Prohibited Method*(s), the Laboratory shall consult the TA about the need to conduct the CP for the blood endocrine *Markers*.

4.2 Confirmation Procedure Requests from the *Testing* Authority, the Passport Custodian, the *Athlete* Passport Management Unit or *WADA*.

The Adaptive Model will also flag abnormally low or variable endocrine *Markers*. However, in such cases the Laboratory will not receive an automatic notification through *ADAMS*. Instead, the *Athlete* Passport Management Unit (APMU) will advise the PC (who will advise the TA, if different) on whether the *Sample*, or other *Samples* from the corresponding Passport, shall be subjected to a CP for the blood endocrine *Markers* and the application of the hGH Isoform Differential Immunoassay. Therefore, in these cases the Laboratory shall receive a written request from the TA[3], or *WADA*, before proceeding with the CP.

4.3 Confirmation of Blood Endocrine *Marker* Values

The CP for the blood endocrine *Markers* following receipt of an Endocrine-CPR, or upon request, consists of the application of the same assay pairing used for the quantification of the concentrations of IGF-I and P-III-NP in the ITP as well as the identification of IGF-I (in compliance with the ISL *TD* IDCR [3]).

4.4 hGH Isoform Differential Immunoassay Requests ("Isoform-Requests") triggered through *ADAMS*

a) Once the CP data of the blood endocrine *Markers* is entered and matched with the corresponding Passport in *ADAMS*, the Adaptive Model automatically updates the endocrine Passport with the confirmed *Marker* values.

b) If the ITP triggers an *Atypical Passport Finding* (*ATPF*) for high IGF-I, which is confirmed in the subsequent CP, an Isoform-Request for the performance of the hGH Isoform Differential Immunoassay (see ISL *TD* GH [4]) is triggered and sent automatically to the Laboratory through *ADAMS* (see Figure 1).

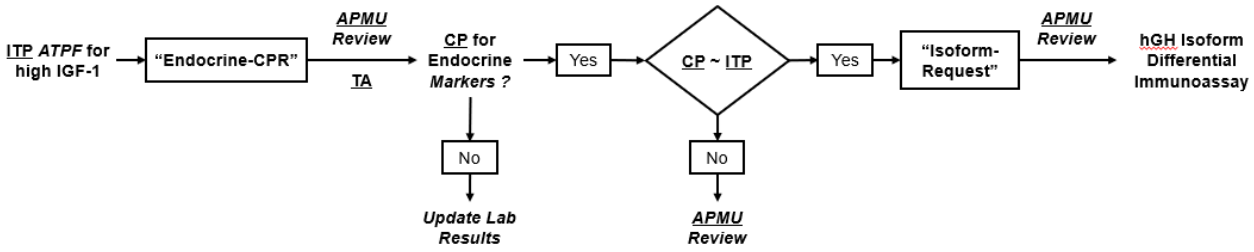

Figure 1: Steps leading to the application of the hGH Isoform Differential Immunoassay to *Samples* with elevated IGF-1 identified in the ITP.

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5.0 Reporting Results for the Blood Endocrine *Markers*

a) The IGF-I and P-III-NP concentrations shall be reported in *ADAMS* in nanograms per milliliter (ng/mL).

[Comment to Article 5.0 a): For the purposes of the Endocrine Module of the ABP, the GH-2000 score does not need to be calculated or reported by the Laboratory; it will be automatically calculated in ADAMS].

b) If the measured concentration(s) of IGF-I and/or P-III-NP is below the LOQ of the assay, the Laboratory shall report a value of "-1" for the affected concentration in *ADAMS*.

c) If the concentration(s) of IGF-I and/or P-III-NP cannot be determined or the *Marker*(s) cannot be identified, the affected *Marker(s)* shall be reported as "-1" and the Laboratory shall make a corresponding comment in the Lab Results in *ADAMS* (*e.g.,* matrix interferences).

6.0 References

[1] The World Anti-Doping *Code International Standard* for Laboratories (ISL).

[2] *WADA Technical Document* ISL *TD* ATP: Analytical *Testing* Procedures.

[3] *WADA Technical Document* ISL *TD* IDCR: Minimum Criteria for Chromatographic-Mass Spectrometric Confirmation of the *Identity* of Analytes for *Doping Control* Purposes.

[4] *WADA Technical Document* ISL *TD* GH: Human Growth Hormone (hGH) Isoform Differential Immunoassays for Doing Control Analyses.

[5] The World Anti-Doping *Code International Standard* for *Testing* (IST).

[6] *WADA Technical Document* ISL *TD* LCOC: Laboratory Chain of Custody.

[7] *WADA Technical Document* ISL *TD* VAL: Minimum Requirements for Validation of Analytical *Testing* Procedures for *Doping Control.*

[8] *WADA Technical Document* ISL *TD* APMU: Athlete Passport Management Unit Requirements and Procedures

[Comment to Article 6.0: Current versions of WADA International Standards and Technical Documents may be found at https://www.wada-ama.org/en/what-we-do/international-standards]